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13. ABSTRACT (Maximum 200 Words) This project aimed to define the effects of green tea polyphenols (GTPs) on cell signaling pathways and on mammary tumorigenesis in MMTV/c-neu transgenic mice. The studies showed that the major polyphenol in green tea, EGCG, did not impair erbB-2 tyrosine phosphorylation or EGF-dependent activation of MAP kinase in transformed or untransformed MCF10A cells, and that orally administered green tea or GTPs did not inhibit mammary tumorigenesis. Subsequent studies focused on the mechanism by which EGCG inhibits mammary epithelial cell cycle progression. EGCG inhibited S phase entry in epidermal growth factor (EGF)-stimulated MCF10A breast epithelial cells when provided in G0 or mid G1, but not when provided after the late G1 restriction point. EGCG induced p21 ^{CIP1/WAF1/SDI1} , inhibited cyclin D1-associated pRB kinase activity, increased the association of p21 with cyclin D1, and impaired pRB phosphorylation. EGCG induced p21 expression at the mRNA level. In addition, the ability of EGCG to induce p21 depended on the addition of EGF, indicating that EGCG synergizes with growth factor-dependent signals. While these findings provide a mechanism by which polyphenolic compounds may inhibit proliferation, their relevance to breast cancer chemoprevention remains unclear due to the lack of effect of tea polyphenols on tumorigenesis in MMTV/c-neu transgenic mice.				
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FOREWORD

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INTRODUCTION

Green tea polyphenols (GTPs) are potentially useful for delaying the onset of breast cancer, since they suppress tumorigenesis in rodents, they inhibit tumor cell proliferation *in vitro* and *in vivo*, and they are apparently safe natural products. However, since the mechanism through which GTPs suppress tumorigenesis is not known, it is unclear how to maximize their efficacy to prevent cancer or how to identify or develop compounds with improved efficacy. Accordingly, the current studies were undertaken to define the mechanism through which green tea polyphenols (GTPs) inhibit cell proliferation, as well as to evaluate their ability to suppress a genetically defined mammary tumor in mice. Studies to determine how GTPs inhibit breast tumor cell proliferation initially evaluated the hypothesis that GTPs impair mitogenic signalling by receptor tyrosine kinases (RTKs) by preventing the accumulation of hydrogen peroxide (H_2O_2) and thus sustaining the activity of tyrosine phosphatases that otherwise diminish RTK phosphorylation and activity. This hypothesis was supported by evidence that GTPs reduce H_2O_2 levels *in vivo*, that H_2O_2 can mediate mitogenic signalling, that H_2O_2 promotes tyrosine phosphorylation of the EGF and insulin receptors, and that H_2O_2 can inhibit tyrosine phosphatases. Additional studies were to focus on the effects of GTPs on the proliferation and RTK phosphorylation of non-transformed MCF10A human mammary epithelial cells, as well as on MCF-10A derivatives expressing human *erbB-2* (MCF10A/*erbB-2*) or a constitutively active rat *c-neu* oncogene (MCF10A/*c-neu*). Studies were also directed towards defining the effects of GTPs on mammary tumorigenesis in MMTV/*c-neu* transgenic mice. These mice express constitutively active *c-neu* and develop mammary adenocarcinoma *in situ* in all transgene-expressing mammary tissue, with palpable tumors formed between 11 and 14 weeks in parous females. The rapid tumor development, nearly uniform timing of tumorigenesis, and genetic identity of the mice make them appropriate for studying the effects of GTPs on *c-neu*-induced oncogenesis. In the event that green tea or GTPs were to inhibit tumorigenesis, it was intended to determine whether such agents affected RTK phosphorylation and cell proliferation or survival.

BODY

This section describes research accomplishments associated with each Task outlined in the **approved** Statement of Work, and provides a complete record of the research findings for the period of the annual/final report.

Original Task 1. To establish GTP concentrations that are needed to inhibit proliferation of MCF-10A and MCF-10A-derived cells treated with EGF, β -NDF, or IGF-I. As detailed in the first Annual Report, this Task was completed in part. EGCG concentrations that were needed to inhibit EGF-dependent proliferation were established (see Appendix). However, since EGCG

concentrations that inhibited EGF-dependent proliferation had no effect on erbB-2 tyrosine phosphorylation or MAP kinase activation, we realized that EGCG was likely to impair proliferation at a step distal to receptor activation and MAPK signalling. Accordingly, we reappraised our strategy for defining GTP anti-mitogenic effects. Rather than focus on selected early signalling events such as H₂O₂ production and RTK phosphorylation, we defined the effects of EGCG on cell cycle progression as a whole, and showed that it blocked progression from the G1 to the S phase of the cell cycle. This was included in the Revised Statement of Work submitted in October 1999, and was pursued as in *New Tasks 5, 6, and 7*.

Original Task 2. To determine whether growth-inhibiting concentrations of GTPs also diminish tyrosine phosphorylation associated with the EGFR, erbB-2, or IGF1R. This task was completed, as indicated in the first Annual Report. We found that EGCG inhibited proliferation of MCF10A, MCF10A/*erbB-2*, and MCF10A/*c-neu* cells to similar extents and that EGCG concentrations that inhibited EGF-dependent proliferation had no effect on the transient tyrosine phosphorylation of erbB-2 or on MAPK activation in EGF-stimulated MCF10A or MCF10A/*c-neu* cells. These findings argued against the hypothesis that GTPs impaired RTK tyrosine phosphorylation and are detailed in the Appendix.

Original Task 3. To establish the effect of growth inhibitory doses of GTPs on H₂O₂ accumulation. As detailed in the first Annual Report, this task was not completed due to the change in experimental approach, as indicated in *Original Task 1*, above.

Original Task 4. To establish the effect of decaffeinated green tea or GTPs on mammary tumor growth, cell proliferation, cell survival, and RTK tyrosine phosphorylation in MMTV/*c-neu* mice. In a pilot study, five mice drinking 1% lyophilized green tea (LGT) developed fewer tumors than the three water-drinking controls, yet the LGT mice exhibited gastrointestinal dysfunction and weight loss that may have underlied the decreased tumorigenesis due to changes in overall caloric balance. In a second study, tumor formation in MMTV/*c-neu* mice was quantitated after drinking water (10 mice), 0.8% LGT (6 mice), decaffeinated lyophilized green tea (6 mice), or GTPs (5 mice). In this study, there was not substantial weight loss among the LGT population, presumably due to the decreased tea concentration and/or more progressive weaning onto tea. However, there was also no significant anti-tumor effect of any of the tea preparations. Two additional cohorts of mice which were progressively weaned onto 1% LGT, starting at 5 weeks of age, or controls provided with water were analyzed for the presence of hyperplastic nodules in mammary tissue whole mounts. In these mice, there was no significant weight difference between the two populations and there was a trend towards an increase in such nodules in 1% LGT drinking mice, although the trend was not statistically significant. All of these studies are presented in detail in the first Annual Report. In summary, the results show that tea polyphenols are not effective chemopreventive agents in MMTV/*c-neu* mice, and they suggested that anti-tumor effects attributed to tea may in some cases result from GI toxicity and weight loss.

New Task 5. To identify changes in cell cycle control that underlie growth inhibition by EGCG. It was shown that EGCG inhibits progression from the G1 to the S phase of the cell cycle in EGF-stimulated cells when provided in G0 or mid G1, but not when provided after the late G1 restriction point. We then evaluated the effects of EGCG provided to mid G1 MCF10A cells on regulators of G1 cell cycle progression. EGCG inhibited the induction of cyclin D1 in early G1 but not in mid G1, and it induced p21^{CIP1/WAF1/SDI1} (hereafter referred to as p21), during all phases of the cell cycle and inhibited retinoblastoma protein (pRB) phosphorylation. These studies are described in detail in the accompanying manuscript in the Appendix.

New Task 6. To determine whether the increase in p21 expression leads to increased association of p21 with the kinases Cdk4 and/or Cdk2, as well as impaired Cdk4 and Cdk2 kinase activity. It was shown that EGCG increased the association of p21 with cyclin D1, and this is believed to reflect binding to cyclin D1:Cdk4 or cyclin D1:Cdk6 complexes. EGCG also inhibited cyclin D1-associated pRB kinase activity. Since p21:cyclin D1:Cdk4,6 complexes may be active, it is believed that the association of p21 with cyclin D1 might not be sufficient for inhibition of cyclin D1-associated pRB kinase, and that other, as yet undiscovered effects may ultimately be responsible for inhibiting cyclin D1:Cdk4. Under most circumstances in which cyclin D:Cdk4-associated pRB kinase activity is impaired, there is also impaired Cdk2 function and this is most likely to be true following EGCG treatment. We have vigorously pursued measuring the effect of EGCG on Cdk2 kinase activity, given that the observed effect on cyclin D:Cdk4 activity is likely the major cell cycle inhibitory effect of the compound. See Appendix for details.

New Task 7. To determine whether EGCG sustains p21 at the RNA level, by increasing p21 transcription, or at the protein level, by increasing p21 translation or protein stability, and to define *cis*-acting elements through which such effects are brought about. Northern blotting analysis showed that EGCG induced p21 expression at the mRNA level. MCF1-10A cells were serum starved and then stimulated with complete media and treated with either EGCG or vehicle, and total RNA prepared for Northern analysis. The results showed that p21 levels rose and then declined in control cultures, whereas p21 mRNA levels declined at a far slower rate following EGCG treatment. Studies to define the *cis* acting sequence elements in the p21 promoter through which EGCG induces p21 expression have been initiated. To do so, we obtained a series of p21 promoter – chloramphenicol acetyl transferase (CAT) reporter constructs containing various extents of p21 promoter sequence. These are being transfected into MCF10A cells and the ability of each to be expressed and induced by EGCG is being examined. However, these studies have not been completed.

In addition to the above work, which reflects Tasks in the revised Statement of Work, we have shown that the ability of EGCG to induce p21 depended on addition of exogenous growth factors (see Appendix). This finding indicates that EGCG synergizes with growth factor-

dependent signals to induce p21 and impair cell cycle progression. We have also investigated whether the induction of p21 by EGCG reflects an ability to impair Rho protein signaling. Since p21 can be repressed by Rho protein signaling, Rho is required for G1 progression in response to growth factors, and Rho is required for the repression of p21 as well as for transformation of by Ras, we reasoned that EGCG may sustain p21 expression, and perhaps interfere with Ras-mediated transformation, by inhibiting the repression of p21 by Rho. To test this concept, we evaluated whether inhibition of Rho signaling by C3 transferase would result in the up-regulation of p21 in MCF10A cells. In initial experiments, we found that cells microinjected with C3 expressed increased levels of p21 as measured by fluorescence microscopy. This sets the stage for future studies focused on the ability of EGCG to inhibit Rho signaling.

KEY RESEARCH ACCOMPLISHMENTS

- EGCG did not impair erbB-2 tyrosine phosphorylation or EGF-dependent activation of MAP kinase in transformed or untransformed MCF10A cells.
- Orally administered green tea or GTPs did not inhibit mammary tumorigenesis in MMTV/*c-neu* mice.
- EGCG inhibited S phase entry in EGF-stimulated cells when provided in G0 or mid G1, but not when provided after the late G1 restriction point.
- EGCG induced p21^{CIP1/WAF1/SDI1}, inhibited cyclin D1-associated pRB kinase activity, increased the association of p21 with cyclin D1, and impaired pRB phosphorylation.
- The ability of EGCG to induce p21 depended on the addition of EGF, indicating that EGCG synergizes with growth factor-dependent signals to induce p21 and impair cell cycle progression.
- EGCG induced p21 expression at the mRNA level.

REPORTABLE OUTCOMES

Manuscript:

Liberto, M., and **D. Cobrinik**. 2000. Growth Factor-Dependent Induction of p21^{CIP1} by the Green Tea Polyphenol, Epigallocatechin Gallate. *Cancer Letts.*, in press. (See Appendix)

CONCLUSIONS

This project was directed towards understanding the mechanism by which green tea polyphenols function as chemopreventive agents. It was initially centered on understanding the effects of such agents on the early steps of growth factor receptor tyrosine kinase signaling. However, we soon determined that this step was not affected by tea polyphenols, and endeavored to define more downstream events that were affected. Accordingly, we showed that EGCG resulted in enhanced, but still growth factor-dependent induction of p21 together with impaired cyclin D1-associated pRB kinase activity. This implies that EGCG and perhaps other tea

polyphenols act at a signaling step downstream from receptor tyrosine kinases that ultimately impinges on p21 control. Since p21 can be repressed by Rho protein signaling, Rho is required for G1 progression in response to growth factors, and Rho is required for the repression of p21 as well as for transformation of by Ras, it seems plausible that EGCG sustains p21 expression, and perhaps interferes with Ras-mediated transformation, and this idea is being pursued. A second Aim of the project was to determine whether EGCG impaired mammary tumorigenesis in MMTV/*c-neu* transgenic mice. As indicated in the first Annual Report, the results clearly showed no substantial chemopreventive effect. These findings argue against the indiscriminate use of green tea and other anti-oxidants as chemopreventive agents, and imply that further mechanistic, animal modeling, and human studies need to be done before such approaches can be rationally employed.

REFERENCES

The concepts described in this Report are embodied in the manuscript by Liberto and Cobrinik (see Appendix), and References are included therein.

APPENDIX

In Press: Cancer Letts. 2000

**Growth Factor-Dependent Induction of p21^{CIP1} by the Green Tea Polyphenol,
Epigallocatechin Gallate**

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ABSTRACT

Tea polyphenols inhibit tumorigenesis and cell proliferation in rodent models, but their effects on cell signaling and cell cycle control pathways are undefined. Here, we show that the major polyphenol in green tea, epigallocatechin gallate (EGCG), inhibits S phase entry in epidermal growth factor (EGF) -stimulated MCF10A breast epithelial cells when provided in G0 or mid G1, but not when provided after the late G1 restriction point. EGCG induced p21^{CIP1/WAF1/SDI1}, inhibited cyclin D1-associated pRB kinase activity, and impaired pRB phosphorylation. The ability of EGCG to induce p21 depended upon the addition of EGF, indicating that EGCG synergizes with growth factor-dependent signals to induce p21 and impair cell cycle progression.

Key Words: tea polyphenols, cyclin D, p21^{CIP1/WAF1/SDI1}, chemoprevention

INTRODUCTION

Current estimates indicate that dietary factors contribute to one-third of annual cancer related deaths in the United States [1]. While the traditional Western diet is believed to contribute to an increased lifetime risk of cancer, diets high in plant-derived foods offer a protective effect [2,3]. Accordingly, an important strategy for cancer prevention is the identification and characterization of dietary phytochemicals that are able to block, slow, or reverse tumorigenesis. Defining the mechanism of action of such compounds may contribute to rational approaches to cancer treatment and prevention.

One of the largest and most ubiquitous groups of phytochemicals is the polyphenols, which may be consumed in quantities as high as 1-2 g/day [4,5]. Polyphenols include many potential chemopreventive agents, especially among the flavonoids and their derivatives. Many of these compounds have been shown to inhibit carcinogenesis in animal models and to impair the proliferation and survival of tumor cells *in vitro* [4]. Although the effects of such polyphenols on human cancer have not been established, recent studies suggest that particular compounds may be protective [6-8].

Tea is a significant source of flavonoids and related polyphenolic compounds with chemopreventive activity, including the ability to inhibit tumorigenesis in rodent models (see [9,10] for reviews). In some carcinogenesis regimens, orally administered tea polyphenols inhibited both tumor initiation and tumor promotion [11]. These activities may be attributed, in part, to an ability to block the damaging effects of carcinogens on cellular DNA [12,13]. However, this blocking ability is unlikely to fully explain the chemopreventive effects of tea polyphenols, since tumorigenesis was also prevented under conditions where there was no inhibition of carcinogen-induced DNA damage [14] or when tea or tea polyphenols were provided after a carcinogenic insult [15-20]. Indeed, epigallocatechin gallate (EGCG), which comprises approximately 50% of the polyphenol fraction of green teas, has been shown to inhibit

the growth of both spontaneous [21] and established tumors [15,22], as well as the growth of tumor cells implanted in nude mice [23].

In addition to their anticarcinogenic effects, tea polyphenols can inhibit cell proliferation and cell survival at various stages during tumorigenesis [16,17,20,24,25]. These activities may be relevant to cancer prevention in experimental regimens where tea polyphenols are provided after a carcinogen. The ability of polyphenols to inhibit mitogenic signaling *in vivo* is supported by *in vitro* studies demonstrating antiproliferative effects of EGCG on numerous cell lines [24,26-35]. However, mechanisms by which EGCG inhibits proliferation have not been established.

In the current study, we have sought to determine how EGCG alters cell cycle control pathways in epidermal growth factor (EGF) stimulated MCF10A mammary epithelial cells. Since treatment with EGF is sufficient to stimulate MCF10A cells to proliferate, it was reasoned that this would provide a useful system with which to dissect the effects of EGCG on a specific cell growth signaling pathway [36]. We report that EGCG inhibits EGF-induced MCF10A cell proliferation accompanied by increased p21^{CIP1/WAF1/SDI1} expression, decreased cyclin D1-associated pRB kinase activity, decreased pRB phosphorylation, impaired progression through the late G1 restriction point, and diminished entry into the S phase of the cell cycle. Notably, induction of p21 by EGCG depended upon the presence of EGF, suggesting that EGCG synergizes with growth factor induced signaling pathways to inhibit proliferation.

METHODS

Chemicals. DMEM:F12 was from MediaTech (Herndon, VA), l-glutamine and antibiotics were from Gibco/BRL (Gaithersburg, MD). Nonidet P-40 and nitrophenyl phosphate were from Boehringer Mannheim (Indianapolis, IN). Unless noted, all other chemical were from Sigma (St. Louis, MO). EGF stock solution (100 ug/ml) was prepared in 10 mM acetic acid and 1% bovine serum albumin and stored at -20° C. EGCG stock solution (50 mM) was prepared in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 (PBS), containing 5 mM glucose and stored at -20° C.

Cell culture and treatments. MCF10A cells were maintained as described previously [37] in DMEM:F12 (1:1) with 5% horse serum, 4 mM l-glutamine, 100 ug/ml penicillin/streptomycin, 20 mM Hepes, 10 ug/ml insulin, 0.50 ug/ml hydrocortisone, 0.10 ug/ml cholera toxin, and 0.02 ug/ml EGF. For all experiments, cells from confluent cultures were seeded into serum-free media consisting of DMEM:F12 (1:1) with 4 mM L-glutamine, 100 ug/ml penicillin, 100 ug/ml streptomycin, 20 mM HEPES, 1 mg/ml BSA, 5 ug/ml transferrin, 0.02 ug/ml sodium selenite, 1 ug/ml hydrocortisone, and 1 mM ethanolamine [38], and supplemented with 2% horse serum. Cells were cultured for 2 days, after which media replaced with serum-free media containing 5 ng/ml EGF without or with EGCG.

Cell proliferation assay. Cells were cultured for three additional days followed by counting of cells in triplicate with a hemocytometer. Trypan blue was used to exclude dead cells.

Cell cycle analysis. Cells were stimulated with serum-free media containing EGF and 30 μM 5-bromo-2-deoxyuridine (BrdU), 30 μM 2-deoxycytidine, and 10 μM 5-fluorodeoxyuridine. Cells were fixed for 30 minutes in ice cold 70% ethanol and DNA denatured by incubation in 2N HCl, 0.5% Triton X-100. Samples were neutralized in 0.1 M sodium tetraborate and then incubated with a monoclonal antibody to BrdU (Becton Dickinson) at 20 $\mu\text{l/ml}$ for 30 minutes. Cells were washed twice in PBS containing 0.5% Tween 20 (PBS-Tween) and then incubated with a fluorescein conjugated goat anti-mouse secondary antibody (Becton Dickinson) at 80 $\mu\text{l/ml}$ for

30 minutes at room temperature, followed by washing and staining in PBS containing 10 ug/ml propidium iodide and 50 ug/ml RNase A. BrdU-positive nuclei were detected using two color flow cytometry with 10^4 gated events collected for each analysis.

Western Blotting. Cells were lysed in 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 5% sodium deoxycholate, and 10% Nonidet P-40, 10 ug/ml pepstatin, 1 mM sodium vanadate, 50 ug/ml leupeptin, 20 ug/ml aprotinin, 100 ug/ml phenylmethylsulfonate, and 15 mg/ml nitrophenyl phosphate. Protein was quantitated using a modified Lowry method (BioRad DC protein assay), separated by SDS-polyacrylamide gel electrophoresis, and electroblotted to a nitrocellulose support. Efficiency of transfer and equal loading of protein were confirmed by staining membranes with 0.1% Ponceau S in 5% acetic acid. Membranes were blocked for 1 hour at room temperature in PBS-Tween and 5% nonfat dry milk, and were then incubated with antibody in PBS-Tween with 2.5% milk either overnight at 4° C or for 1 hour at room temperature, washed in PBS-Tween three times for 10 minutes at room temperature, and developed with an enhanced chemiluminescence kit (Kirkegaard & Perry, Gaithersburg, MD). The following primary antibodies were used: cyclin D1 (ms-210) and p21^{CIP1} (ms-230) from LabVision (Fremont, CA); cyclin E (sc-247), p27^{KIP1} (sc-528), and p53 (sc-126) from Santa Cruz Biotechnology (Santa Cruz, CA); pRB (14001A) from Pharmingen (San Diego, CA); ERK1/2 (9102); phospho-specific ERK1/2 (9101) from New England Biolabs (Beverly, MA); and horseradish peroxidase conjugated secondary antibodies from Santa Cruz Biotechnology.

IP-kinase assay. Assays were performed as described [39] with some modifications. Cells were harvested by scraping in Hepes-buffered Hanks balanced salt solution on ice, collected by centrifugation, suspended in lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween-20, and 10 % glycerol, 1 mM Pefabloc (Boehringer Mannheim), 1 mM sodium vanadate, 5 ug/ml leupeptin, 2 ug/ml aprotinin, 10 mM beta-glycerophosphate), and sonicated for 10 seconds. The lysate was cleared by centrifuging twice for 10 minutes each at 14,000 x g. 200 ug protein (quantitated by Bradford method, BioRad) was mixed with 1 ug anti-cyclin D1 antibody DCS-11 (LabVision) per 100 ul, rotated at 4° C for

1.5 hours, added to 20 ul of pre-washed Protein G beads (Pharmacia), incubated for an additional 1.5 hours at 4° C, and washed three times with 1 ml lysis buffer and twice with 250 ul kinase buffer (50 mM Hepes, 10 mM MgCl₂, 1 mM DTT, 20 mM para-nitrophenyl-phosphate, 20 mM beta-glycerophosphate). Beads were suspended in 30 ul kinase buffer containing 1 ug Rb substrate (Santa Cruz) and incubated for 5 minutes at room temperature. 35 uM unlabeled ATP and 5 uCi γ -³²P-ATP was added to each tube and samples incubated for 30 minutes at 30° C, followed by addition of 10 ul sample loading buffer, boiling, and SDS-polyacrylamide gel electrophoresis. The gel was fixed for 30 minutes in 40% isopropanol, 10% acetic acid, stained for 15 minutes in 0.006% Coomassie Blue to ensure equal protein loading, fixed in 10% acetic acid overnight, dried for 1 hour at 80° C, and visualized by autoradiography.

RESULTS

EGCG impairs MCF10A cell proliferation and progression through the late G1 restriction point. To identify the cell cycle effects of EGCG, we first determined the concentration range of EGCG that inhibited EGF-induced MCF10A cell proliferation and the phase of the cell cycle in which proliferation was blocked. Cells were arrested by culturing for two days in low serum, which was sufficient for at least 90% of the cells to accumulate in G0/G1 as measured by flow cytometry (data not shown). The plating media was then replaced with serum-free media containing EGF alone or in combination with EGCG, and cells were counted after an additional three days. As shown in Fig. 1A, EGCG inhibited MCF10A proliferation in a dose-dependent manner between 25 and 100 μ M. Dead cells accounted for less than 5% of the total in all treatment groups (not shown), indicating that EGCG inhibited proliferation without cytotoxicity.

Since the primary mitogenic effect of EGF is to promote S phase entry, we examined whether EGCG opposed this process. Growth arrested MCF10A cells were stimulated with EGF alone or in combination with EGCG, and the S phase population was labeled with 5-bromo-2-deoxyuridine (BrdU) and detected by flow cytometry 18 hours after stimulation. This was approximately 4 hours after stimulated cells first entered S (data not shown). As shown in Fig. 1B, EGCG inhibited S phase entry at concentrations similar to those that inhibited proliferation, suggesting that the effect on S phase entry contributed to growth inhibition.

The G1 phase of the cell cycle can be divided into growth factor-dependent and growth factor-independent segments. The restriction point transition between these segments is a critical regulatory point during which a cell commits to cell cycle entry. To determine whether EGCG acts during the growth factor-dependent portion of G1, we determined whether EGF-stimulated MCF10A cells were sensitive to EGCG at times when they required EGF, but insensitive to EGCG at times when they no longer required EGF for S phase entry. As shown in Fig. 2A, the proportion of MCF10A cells entering S phase increased progressively as EGF was allowed to remain in the media for longer times. Approximately 66% of normal S entry occurred when

EGF was withdrawn 10 hours after stimulation, indicating that this proportion of cells had completed the late G1 restriction point transition. As shown in Fig. 2B, EGCG maximally inhibited S phase entry when provided 4 hours after EGF, but had a minimal effect when administered 10 hours after stimulation. Thus, most MCF10A cells became insensitive to EGCG as they approached or passed through the late G1 restriction point.

EGCG impairs pRB phosphorylation and induces p21 expression in EGF-treated cells. The phosphorylation of pRB during G1 is a growth factor-dependent process that is thought to promote restriction point passage [40]. To determine whether EGCG impaired pRB phosphorylation, growth arrested and restimulated cells were treated with EGCG during mid G1, and pRB phosphorylation was monitored by western blotting for electrophoretically distinct phosphorylated pRB species (Fig. 3). In cells treated with EGF alone, slower migrating phosphorylated forms of pRB were evident at 5 hours and continued to accumulate at later times. EGCG provided at 5 hours after stimulation reduced the level of phosphorylated pRB at the 8 and 12 hour time points. Thus, EGCG inhibited the mid G1 accumulation of phosphorylated pRB.

pRB phosphorylation is regulated by G1 cyclin dependent kinases (CDKs) and their inhibitors (CDKIs). Phosphorylation is thought to be initiated by cyclin D-CDK4 or cyclin D-CDK6 and to be completed by cyclin E-CDK2 [41]. Growth factor stimulation generally induces the accumulation of D-type cyclins and reduces expression of the p27 CDKI [42], and in some instances leads to transient induction of p21^{CIP1/WAF1/SDI1} (hereafter referred to as p21) [43,44]. To determine whether EGCG inhibited pRB phosphorylation by disrupting G1 CDK activity, we first examined its effect on the expression of G1 cyclins and CDKIs.

In preliminary studies, MCF10A cells were found to express cyclin D1 but not detectable levels of cyclins D2 or D3 (not shown). In response to EGF alone, cyclin D1 was strongly induced at 4 hours, and addition of EGCG concurrently with EGF reduced cyclin D1 accumulation (Fig. 4A). However, EGCG administered during mid G1, when it maximally

inhibited S phase entry, did not significantly affect cyclin D1 expression (Fig. 4B). Thus, although EGCG inhibited the initial accumulation of cyclin D1, this effect was not sufficient to explain its inhibition of S phase entry. Similarly, cyclin E expression increased within 5 hours after EGF stimulation, remained relatively constant through the remainder of G1, and was not affected by EGCG treatment (Fig. 4C).

We next examined whether EGCG altered the expression of the p21 and p27 CDKIs. As shown in Fig. 5A, p21 transiently increased after EGF stimulation of MCF10A cells, peaking at 2 hours and then decreasing to basal levels by 4 hours. When EGCG was administered concurrently with EGF, the induction of p21 was enhanced, and p21 expression was maintained at substantially higher levels at the 4 hour time point as well as at later times in G1 (Fig. 5A and data not shown). The effect of EGCG was dose-dependent between 25 and 100 μ M (Fig. 5B), corresponding to concentrations that inhibited S phase entry (see Fig. 1). Moreover, when EGCG was administered 5 hours after stimulation with EGF, p21 expression increased within 3 hours and remained approximately three-fold higher through the 12 hour time point (Fig. 6A). Remarkably, EGCG did not induce p21 when it was provided to growth arrested MCF10A cells in the absence of EGF (Fig. 5C), indicating that the induction of p21 by EGCG required an EGF-dependent signal.

In contrast to the effect of EGCG on p21, addition of EGCG in mid G1 did not affect the expression of p27. As observed in other cell types [42], p27 was high in G0 and early G1, and decreased substantially in mid to late G1 regardless of EGCG treatment (Fig. 6B).

Induction of p21 by EGCG correlates with impaired cyclin D1-associated pRB kinase activity. The ability of EGCG to induce p21 in mid and late G1 cells suggests that this effect may play a significant role in impairing CDK-dependent pRB phosphorylation and blocking S phase entry. Since pRB phosphorylation is initiated by cyclin D-dependent kinases, we examined whether EGCG inhibited cyclin D1-dependent pRB kinase activity. EGCG was added to cultures 5 hours after EGF, and at one and three hours thereafter cyclin D1 was

immunoprecipitated, and the associated pRB kinase activity was monitored. As shown in Fig. 7, cyclin D1-associated pRB kinase activity decreased at both the 6 and the 8 hour time points. In separate experiments, the association of p21 with cyclin D1 was found to increase after EGCG treatment (data not shown). These findings support the notion that the EGCG-induced increase in p21 expression led to increased association of p21 with cyclin D1 and impaired pRB kinase activity.

EGF-dependent induction of p21 is not associated with sustained activation of ERK1/2.

The finding that the induction of p21 by EGCG depended upon EGF stimulation suggested that EGCG might enhance a growth factor-dependent signaling pathway that is linked to p21 expression. Recently, p21 was shown to be induced in response to activated Ras and Raf. This induction depended upon activation of extracellular regulated kinases 1 and 2 (ERK1/2), the terminal kinases in the growth factor initiated MAP kinase pathway [45,46]. Accordingly, we investigated whether induction of p21 by EGCG coincided with ERK1/2 activation by measuring expression of the active phosphorylated ERK1/2 with antibodies specific to these species.

As shown in Fig. 8, activation of ERK1/2 in response to EGF followed a characteristic pattern, with ERK1/2 becoming phosphorylated at 1 hour and returning to basal levels of phosphorylation 3 hours after stimulation. EGCG did not alter the amount of phosphorylated ERK1/2 at 1 or 3 hours after EGF stimulation. In addition, EGCG did not induce ERK1/2 phosphorylation when provided in mid G1, after ERK phosphorylation had fallen to basal levels (data not shown). These findings indicate that the induction of p21 by EGCG was not mediated through changes in ERK1/2 activity.

DISCUSSION

In most cell types, growth factor stimulation alters the balance of cell cycle regulatory proteins in a manner favorable to progression through the G1 phase of the cell cycle. Growth factors are required continuously to effect these changes until the cells have passed through the late G1 restriction point, after which S phase can be initiated in the absence of exogenous growth factor signals. This restriction point transition is correlated with specific changes in cell cycle regulatory proteins, including the accumulation of G1 cyclins, a decrease in cyclin dependent kinase (CDK) inhibitors, the activation of G1 CDKs, and phosphorylation of the retinoblastoma protein, pRB [40] .

In this study, we show that EGCG inhibits progression through the restriction point in EGF stimulated MCF10A cells. EGCG was maximally effective when administered in mid G1, but had relatively little effect in late G1 following the transition from EGF dependence to EGF independence that defines restriction point passage. The inhibition of S phase entry by EGCG coincided with increased expression of the p21 CDK inhibitor, increased association of p21 with cyclin D1, impaired cyclin D1-associated pRB kinase activity, and decreased pRB phosphorylation. Importantly, EGCG induced p21 in mid and late G1 cells, when provided either concurrently with EGF or after EGF stimulation. These findings imply that EGCG inhibited proliferation at least in part by increasing p21 expression and thereby inhibiting cyclin D1-mediated pRB phosphorylation. In addition to its effects on p21, EGCG also impaired the initial induction of cyclin D1 by EGF. However, this effect seems unlikely to have contributed to growth inhibition, since cyclin D1 levels were not altered by EGCG in mid or late G1 cells.

While the induction of p21 coincided with impaired cyclin D1-associated pRB kinase activity, it is not clear that kinase inhibition directly resulted from the interaction of p21 with cyclin D1-CDK complexes. Although p21 may directly inhibit cyclin D-associated kinases in particular contexts [49] , recent evidence suggests that p21 fails to inhibit cyclin D-CDK4 and cyclin D-CDK6 when bound to such complexes at a 1:1 ratio [42] . Accordingly, in EGF-

stimulated MCF10A cells, p21 may inhibit cyclin D1-associated pRB kinase activity cells through indirect mechanisms, perhaps by inhibiting the activating phosphorylation of CDK4/6 by CDK activating kinase [47,48] . Aside from its effects on cyclin D1-associated kinases, p21 is expected to inhibit cyclin E-CDK2 through direct interactions, and this may also contribute to the impaired phosphorylation of pRB as well as growth inhibition after EGCG treatment.

In addition to the effects of EGCG on MCF10A cells reported here, the proliferation or survival of other cell lines has also been shown to be inhibited by EGCG and other flavonoids [23,25,26,33,50-53] . A number of these, including genistein, silymarin, silibinin, apigenin, flavone, and EGCG have recently been shown to induce a cell cycle arrest accompanied by increased p21 expression [52-57] . In addition, genistein increased the expression of p21 in tumor cell xenografts, coinciding with a decrease in tumor size [52,58] , suggesting that induction of p21 may be relevant to the chemopreventive effects of such compounds. Our data showing that EGCG inhibits cyclin D-dependent pRB kinase activity, pRB phosphorylation, and cell cycle progression while maintaining high levels of p21 supports a role for p21 in the antiproliferative, and perhaps the chemopreventive activity of these dietary flavonoids. However, chemoprevention by EGCG and related compounds may also relate to their abilities to inhibit MAP kinase signaling pathways and cell transformation in other contexts [59] .

The mechanism through which EGCG increases the expression of p21 is currently unknown. Induction of p21 was not associated with increased expression of p53 (data not shown), suggesting that this regulator of p21 was not central to p21 expression. As a potential clue to the signaling pathways that mediate the EGCG effect, we found that induction of p21 required concurrent EGF stimulation. This implies that EGCG synergizes with a growth factor dependent signaling pathway that is linked to p21 expression. Importantly, the induction of p21 by EGCG was not accompanied by sustained activation of ERK1/2, which contributes to the induction of p21 by mitogens in other contexts [45,46]. From this we conclude that EGCG increases p21 either by enhancing a mitogen-dependent signaling pathway distinct from the Ras - Raf - MAP kinase pathway or by inhibiting a process through which p21 is repressed. Recently,

it was shown that p21 can be repressed by Rho protein signaling, that Rho is required for G1 progression in response to growth factors [60,61] , and that Rho is required for the repression of p21 as well as for transformation of cells that express oncogenic Ras [62,63] . Thus, EGCG may sustain p21 expression, and perhaps interfere with Ras-mediated transformation, by inhibiting the repression of p21 by Rho. This possibility is currently under investigation.

Regardless of the precise mechanism by which EGCG sustains p21 expression, its ability to do so enables it to affect a key mediator of the cell's response to mitogenic signals. The sustained, high level activation of the Ras/ERK pathway has been shown to induce p21 and to cause G1 arrest [45,64] . However, in cases where high level Ras/ERK signaling results in proliferation and transformation, repression of p21 is necessary [62,63] . The results described here suggest that chemopreventive agents such as EGCG have the ability to alter the cell's response to growth factor stimulation by sustaining mitogen-dependent p21 expression and impairing passage through the late G1 restriction point.

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FIGURE LEGENDS

Figure 1. EGCG inhibits EGF-induced cell proliferation and S phase entry. A, cells were stimulated with EGF and the indicated concentration of EGCG, and the cell number determined after incubation for three days. Values are averages of triplicate measurements. B, the indicated concentration of EGCG was added to cells along with EGF and BrdU, and BrdU-positive nuclei were quantitated by flow cytometry after 18 hours. Measurements from two plates were averaged and normalized to the number of BrdU-positive nuclei in the absence of EGCG. Error bars (A, B) represent minimum and maximum values.

Figure 2. MCF10A cells become insensitive to EGCG as they progress through the late G1 restriction point. A, cells were stimulated with EGF for the indicated times, after which EGF was removed and cells cultured in serum-free medium. BrdU-positive nuclei were quantitated 22 hours after the initial stimulation. Values are averages from three experiments and error bars represent standard deviation. B, EGCG was added to EGF-stimulated cells at the indicated times and BrdU-positive nuclei measured at 22 hours. Values are averages from two experiments and error bars represent minimum and maximum values.

Figure 3. EGCG inhibits the phosphorylation of pRB during G1. Cells were stimulated with EGF in the presence (+) or absence (-) of EGCG added at 5 hours. Lysates were collected at the indicated times and analyzed by western blotting. Hypophosphorylated pRB and hyperphosphorylated ppRB are indicated.

Figure 4. Effect of EGCG on expression of G1 cyclins. A, cells were stimulated with EGF in the presence (+) or absence (-) of 50 μ M EGCG, and cyclin D1 expression was monitored by western blotting. B and C, cells were stimulated with EGF; EGCG was added at 5 hours where indicated (+); and cyclin D1 or cyclin E were detected by western blotting at the indicated times.

Figure 5. Effect of EGCG on p21 expression in EGF-stimulated cells. A, cells were stimulated with EGF alone (-) or with EGF in the presence of 50 uM EGCG (+). Lysates were collected at the indicated times and p21 was detected by western blotting. B, cells were treated as in A, except the indicated concentration of EGCG was added together with EGF. C, cells were treated with 50 uM EGCG in the absence of EGF (lanes 2, 4, 5, 6) or with EGF in the absence of EGCG, as a positive control (lane 3). Protein was collected at the indicated times and analyzed by western blotting.

Figure 6. EGCG induces p21 but not p27 in mid G1 cells. Cells were stimulated with EGF alone (-) or with EGCG added at 5 hours (+). Protein was collected at the indicated times and analyzed by western blotting with antibody against either p21 (A) or a p27^{KIP1} (B). Numbers below Panel A indicate integrated pixel density quantitated with NIH Image.

Figure 7. EGCG inhibits cyclin D1-associated pRB kinase activity. Cells were stimulated with EGF alone (-) or with EGCG added at 5 hours (+). Protein was collected at the indicated times, immunoprecipitated with anti-cyclin D1 antibody, and assayed for pRB kinase activity.

Figure 8. Effect of EGCG on EGF-induced phosphorylation of ERK1/2. Cells were stimulated with EGF alone or with the indicated concentration of EGCG. Lysates were collected at the indicated times and activated ERK1/2 analyzed by western blotting with antibodies specific to phosphorylated ERK1/2 (top), followed by blotting with antibodies that detect both the phosphorylated and unphosphorylated ERK1/2 species (bottom).

Figure 1

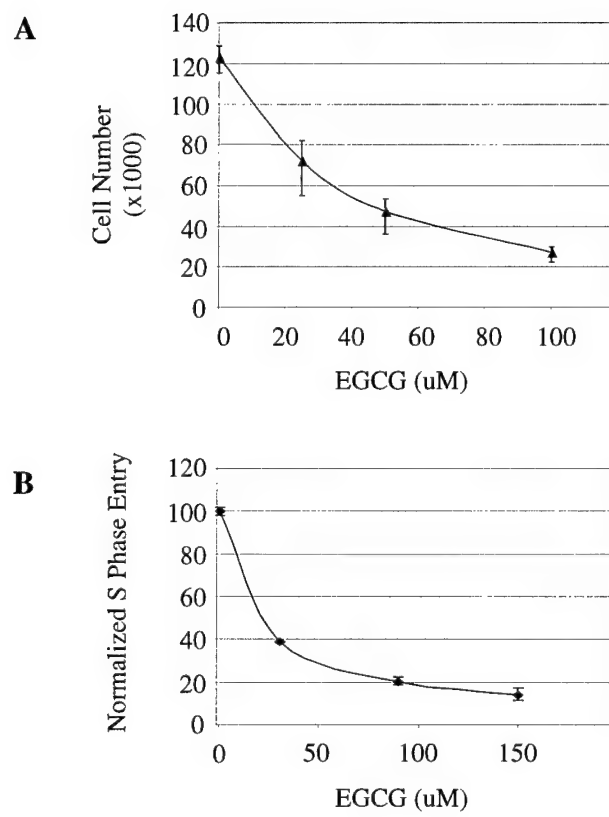


Figure 2

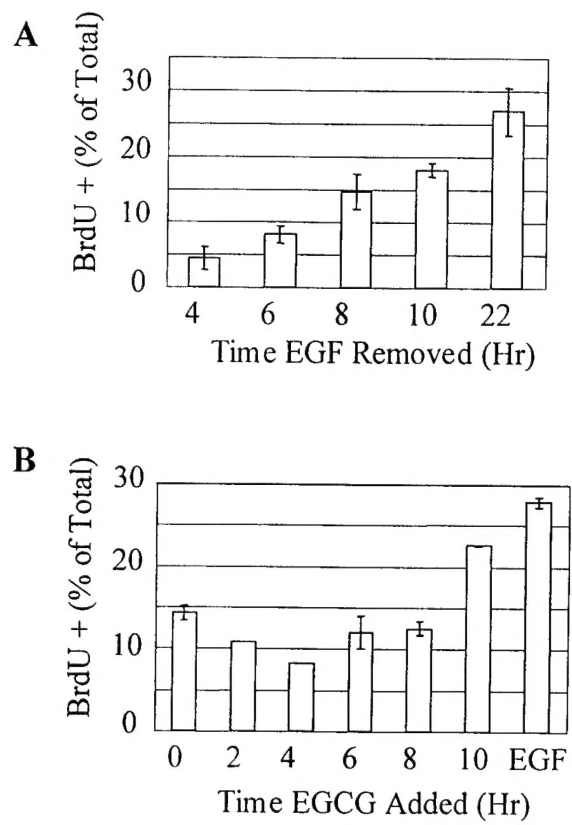


Figure 3

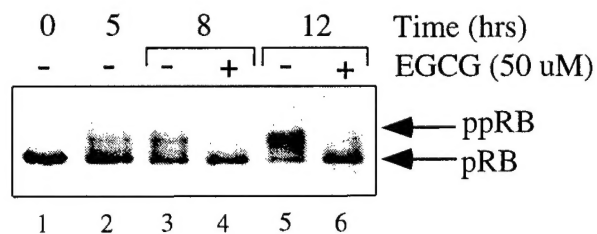


Figure 4

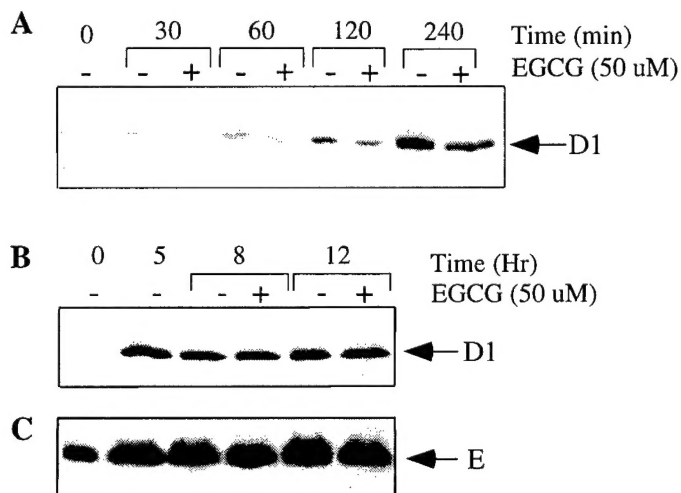


Figure 5

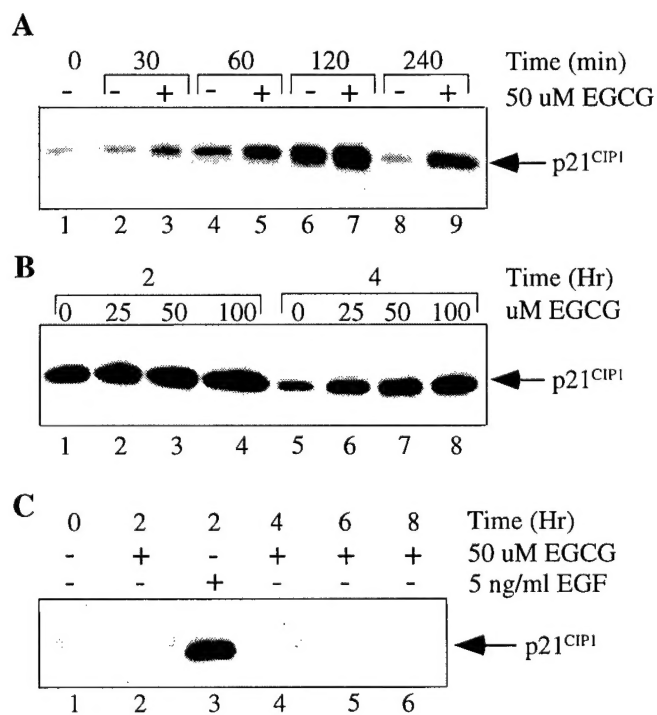


Figure 6

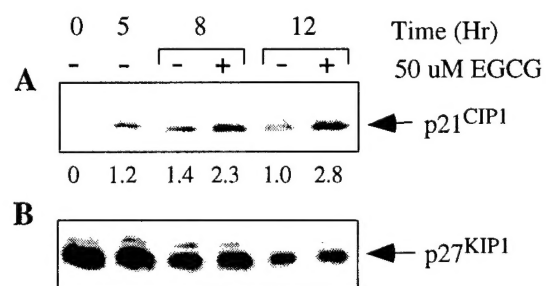


Figure 7

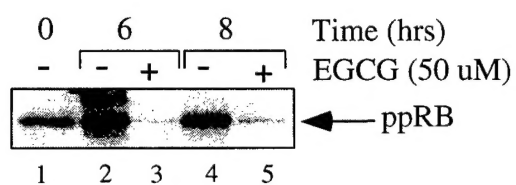
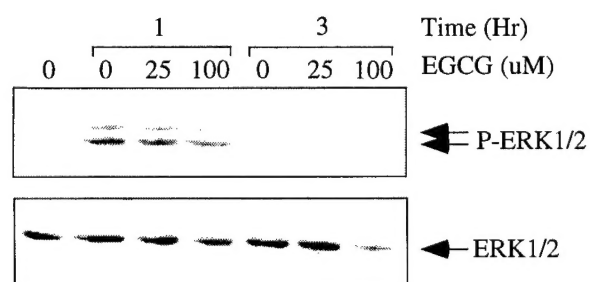


Figure 8



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